PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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| In re Application of: | ) | |
| | ) | |
| Kevin John SLATER *et al* | ) | Examiner: TBA |
| | ) | |
| Serial No.: 10/825,607 | ) | Group Art Unit: 1645 |
| | ) | |
| Filed: April 16, 2004 | ) | Attorney Docket No. 004730.00015 |

For: ASSAY METHODS AND MATERIALS

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Sir:

Submitted herewith is a certified a copy of Patent Application 0308829.1 which was filed in Great Britain on April 17, 2003. This application is the basis for Applicants claim for priority, which claim was made upon filing of the above-identified patent application on April 16, 2004.

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Respectfully submitted,

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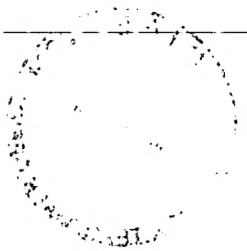
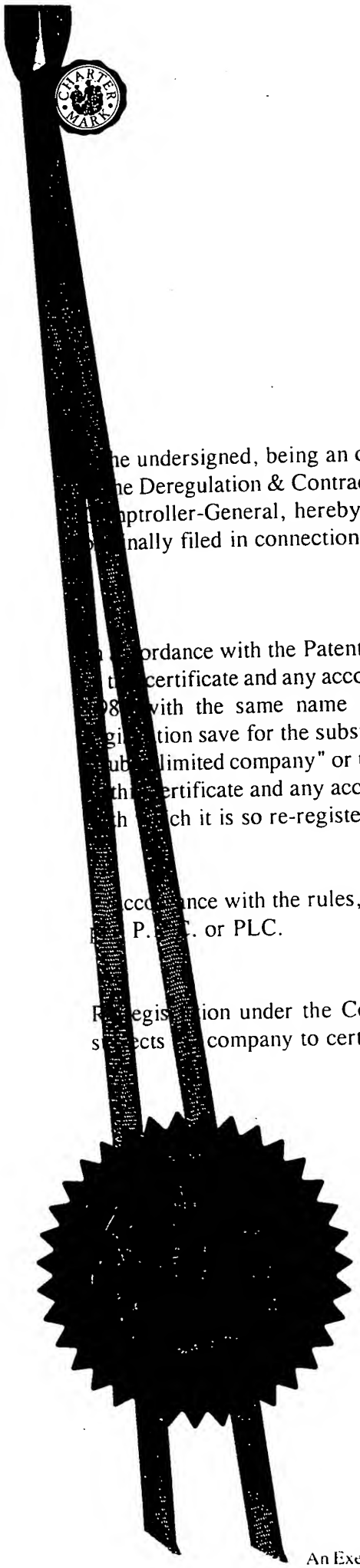
Dated: August 26, 2004





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1. Your reference — CAMT / P27996GB

2. Patent application number *(The Patent Office will fill in this part)* — 0308829.1 — 17 APR 2003

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

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17APR03 E800857-1 D02966
P01/7700 0.00-0308829.1

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation — United Kingdom

8613960501

4. Title of the invention

ASSAY METHODS AND MATERIALS

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Patents ADP number *(if you know it)* — **1305010**

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| Country | Priority application number *(if you know it)* | Date of filing *(day / month / year)* |
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7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

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a) *any applicant named in part 3 is not an inventor; or*
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11. I/We request the grant of a patent on the basis of this application.

Signature Eric Potter Clarkson

**ERIC POTTER CLARKSON**

Date 17 April 2003

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DUPLICATE



## ASSAY METHODS AND MATERIALS

The present invention relates to assay methods and materials for detecting members of the Mollicutes family, that contaminate a test sample, such as a sample from a cell culture.

Taxonomically, the lack of cell walls has been used to separate Mollicutes from other bacteria in a class named *Mollicutes* (Razin *et al* 1998). The members of this class are summarised in the following table 1.

**Table 1: Major Characteristics and Taxonomy of the Class *Mollicutes*.**

| Classification | No. Species | Genome Size (kb) | Mol % G+C of genome | Habitat |
|---|---|---|---|---|
| Order I: *Mycoplasmatales* | | | | |
| Family I: *Mycoplasmataceae* | | | | |
| Genus I: *Mycoplasma* | 102 | 580-1,350 | 23-40 | Humans, animals |
| Genus II: *Ureaplasma* | 6 | 760-1,170 | 27-30 | Humans, animals |
| Order II: *Entoplasmatales* | | | | |
| Family I: *Entoplasmataceae* | | | | |
| Genus I: *Entomoplasma* | 5 | 790-1,140 | 27-29 | Insects, plants |
| Genus II: *Mesoplasma* | 12 | 870-1,100 | 27-30 | Insects, plants |
| Family II:*Spiroplasmataceae* | | | | |
| Genus I: *Spiroplasma* | 33 | 780-2,220 | 24-31 | Insects, plants |
| Order III: *Acholeplasmatales* | | | | |
| Family I: *Acholeplasmataceae* | | | | |
| Genus: *Acholeplasma* | 13 | 1,500-1,650 | 26-36 | Animals, some plants, insects |
| Order IV: *Anaeroplasmatales* | | | | |
| Family: *Anaeroplasmataceae* | | | | |
| Genus I: *Anaeroplasma* | 4 | 1,500 – 1,650 | 29-34 | Bovine/ovine rumen |
| Genus II: Asteroplasma | 1 | 1,500 | 40 | Bovine/ovine rumen |
| Undefined (1999) | | | | |
| *Phytoplasma* | | 640-1,185 | 23-29 | Insects, plants |

In the context of the present application, the term "mycoplasma" is intended to embrace all members of the class *Mollicutes*, not just *Mycoplasmatales*. In fact, "mycoplasma" is the common term in the art for all of the Mollicutes.

Mycoplasmas are widespread in nature as parasites of humans, mammals, reptiles, fish, arthropods and plants. They are the smallest and simplest prokaryotes. They lack a rigid cell wall and are incapable of peptidoglycan synthesis; they are therefore not sensitive to antibiotics, such as penicillin and its analogues. Mycoplasma have developed by degenerate evolution from gram-positive bacteria with a low molecular percentage guanine and cytosine content of DNA ie. the *Lactobacillus, Bacillus, Streptococcus* and two *Clostridium* species. The Mollicutes have lost, during the process of evolution, a substantial part of their genetic information. It is this limited coding capacity that has dictated the need for a parasitic way of life. Most species are facultative anaerobes, but some are obligate, and hence the similarities in their metabolism to anaerobic bacteria.

More than 180 Mollicute species have been identified of which 20 distinct *Mycoplasma* and *Acholeplasma* species from human, bovine and swine have been isolated from cell culture. There are six species that account for 95% of all mycoplasma infections; these are *M. orale, M. arginii, M. fermentans, M. salivarum, M. hyorhinis* and *A. laidlawii*. The major cause of infection is cross contamination from other cell lines introduced into laboratories. Also an unwanted source of exogenous mycoplasma can be found in tissue culture reagents, such as serum products. Mycoplasma, unlike bacterial, contamination rarely produces turbid growth or obvious cell damage. Viable mycoplasma can be recovered from work surfaces seven days after inoculation, and mycoplasma can also pass through bacteria-retaining filters. At their maximum population phase there can be as many as $10^8$ mycoplasma/ml of supernatant, at a ratio of 5:1 with the

host cells. If present, mycoplasma 'grow' to detectable concentrations in the culture medium, they are then also adsorbed onto the cell surface. It is a moot point as to whether mycoplasma enter and survive within mammalian cells in culture.

Mycoplasma are capable of altering almost every property of an *in vitro* culture. They will deplete culture nutrients, in particular arginine. Infected eukaryotic cells exhibit aberrant growth, changes in metabolism and morphology. Certain biological properties have been implicated as virulence determinants; these include secretion or introduction of mycoplasmal enzymes such as phospholipases, ATPases, hemolysins, proteases and nucleases into the host cell milieu.

Previous attempts have been made to use the biochemical properties of mycoplasma to distinguish them from bacteria (reviewed by Rottem and Barile 1993). Originally this focussed around the need for sterol and related cholesterol like compounds; while mycoplasma lack a cell wall, they have a unique cell membrane that contains sterols which are not present in either bacteria or viruses. None of the mycoplasmas tested so far are capable of sterol synthesis, therefore sterol dependence for growth was used to differentiate Mollicutes from other microorganisms. However, this has been shown not to be exclusive, as *Acholeplasma* and *Ureaplasma* have no requirement for sterol (Rottem 2002), and therefore has been discontinued as a diagnostic test . There is currently no single trait to distinguish all mycoplasma from bacteria.

A major problem with mycoplasma is that their contamination is often covert, and unlike bacterial detection, cannot be easily visualised. Their resistance to antibiotics and ability to pass through normal bacterial sterilisation filters means that they can evade typical precautions of cell culture technique. As a result of the negative impact of having these

contaminations going undetected, it has become evident that continuous screening is essential for any cell culture laboratory.

There are a number of studies that have shown that at least 10%-15% of cells in culture may be contaminated with mycoplasma. (Rottem and Barile 1993, McGarrity and Kotani 1985). Most cell biologists recognise the need to perform routine testing for mycoplasma, however due to the cost and inaccuracies of the currently available tests, this has so far remained an unrealised ideal.

The only accurate method available for the detection of viable mycoplasma is culture of the micro-organisms. However, the difficulty associated with their *in vitro* culture has proved problematic due to the complex media required for their cultivation (Razin *et al* 1998). Culture has also been considered to be the most sensitive method, as it is said to be able to detect a single viable organism. However, the results take two to three weeks by highly skilled staff with very specific culture requirements. The time taken is a result of the need to culture the cells to a sufficient number whereby they form colonies, which can then be distinguished using a Dienes stain. Mycoplasma can be cultured on agar and in broth culture, with most mycoplasma producing microscopic colonies with a characteristic 'fried egg' appearance, growing embedded in the agar, although some colonies may not grow completely embedded. There are some strains that cannot be readily grown using standard agar or broth culture media. These strains require cell-assisted culture for their isolation and identification. The latter approach aids in the identification and detection of mycoplasma species that adsorb to host cell surfaces (Rottem and Barile 1993). However, due to the complicated nature of the culturing procedures, these tests are most commonly done by mycoplasma testing service laboratories.

One of the simpler means of detecting mycoplasma in samples is the assay of DNA using a fluorochrome. One of the most commonly used is 4',6-diamine-2-phenylindole dihydrochloride (DAPI), but Hoecsht staining is considered to be the method of choice. Cell culture samples are taken, fixed and stained with Hoechst 33258 (bisbenzamide) and examined under UV epifluorescence (Battaglia et al 1994, Raab 1999). If there are mycoplasma associated with the cells, then the cell nuclei will appear surrounded by fluorescing structures in the cytoplasm. Negative cells are represented by just the nuclear staining of the cellular DNA. Accurate interpretation of results from DNA staining requires an experienced eye, it also needs specialist equipment i.e. a fluorescence microscope.

Mycoplasma detection by PCR is a commonly used test by external service laboratories, and is also performed in those laboratories that have the appropriate equipment. The primers used in mycoplasma PCR kits anneal to conserved regions of the mycoplasma genome, allowing the detection of several species (Raab 1999). Most commercially available PCR kits require that the amplified products be analysed by agarose gel electrophoresis, with the resulting banding patterns determining the contaminating species present. However visualisation of banding patterns is subjective.

The Mycoplasma PCR ELISA from Roche (Raab 1999) relies on a different system, and cannot distinguish between species. This kit includes digoxigenin-dUTP, and the PCR product is captured onto the surface of wells in a microtitre plate coated with anti-digoxigenin-peroxidase conjugate. The coloured product with tetramethylbenzidine (TMB) is visualised using a standard ELISA plate reader.

Life Technologies has developed the MYCOTECT™ Kit, based on the activity of adenosine phosphorylase, which is found only in small amounts (if at all) in mammalian cells (Verhoef *et al* 1983). This enzyme converts

6-methylpurine deoxiriboside (6-MPDR) into two toxic products (6-methylpurine and 6-methylpurine riboside). The assay requires addition of the contaminated cell line to an indicator cell line grown in a 24 well tissue culture plate. The 6-MPDR substrate is added and after 3-4 days of additional growth, a crystal violet stain is added to test for viability of the indicator cells, in that mycoplasma positivity results in production of these toxic agents. Although it has been reported to detect 1 mycoplasma cell per 200,000 target cell, if the medium conditions are adjusted to favour the growth of mycoplasma (Whitaker et al 1987), the main disadvantage of this system is that it is labour intensive and time consuming.

It is possible to detect mycoplasma antigens using immunoassays, employing antibodies raised against mycoplasma antigens. For example, the detection of *M.pneumoniae* in clinical samples (Daxboeck *et al* 2003) Use of different antibodies allows for species identification. There are a number of commercially available kits, for example IDEXX laboratories (US), supply enzyme linked immunosorbent assays (ELISA) for the detection of a number of mycoplasma that have implications in animal health.

Most of the known assays take a minimum of 24 hours to complete, need expensive equipment and a significant amount of expertise. Also, they are strain-specific assays. None are generic, that is, have the ability to detect mycoplasma species in general.

UK patent No. 2 357 336 B describes an assay which can be used to detect mycoplasmas in cell cultures. The assay is based on the observation that mycoplasmas over-produce the enzyme ATPase in large amounts. The ATPase activity of mycoplasmas converts sufficient cellular or externally added ATP to ADP, to make the ADP detectable. Hence, the assay is based on detection of ADP and this is carried out by adding to the sample an enzyme containing reagent (containing a combination of pyruvate kinase

and phosphoenol pyruvate; adenylate kinase; glycerol kinase, myokinase; or a combination of creative kinase and creative phosphate), which converts the ADP to ATP and detecting ATP using a bioluminescent reaction.

The disclosure of UK patent No. 2,357,336 is incorporated herein, including for the purpose of possible amendment.

The present invention seeks to provide further means for detecting mycoplasmas in samples, such as samples from cell cultures.

According to a first aspect the invention provides a method of detecting the presence of mycoplasma in a test sample comprising:

(i) providing a test sample; and

(ii) detecting and/or measuring the activity of acetate kinase and/or carbamate kinase in the test sample, said activity being indicative of mycoplasma contamination.

Preferably, the method further comprises the steps of:

(iii) obtaining acetate kinase and/or carbamate kinase activity information detected and/or measured in a corresponding control sample;

(iv) comparing the activity detected and/or measured in the test sample with that in the control sample; and

(v) identifying the test sample as contaminated with mycoplasma if the activity detected and/or measured in the test sample is greater than that of the control sample.

In a second aspect the invention provides a method of detecting the presence of mycoplasma in a test sample comprising:

(a) providing a test sample; and

(b) detecting and/or measuring the appearance and/or disappearance of one or more of the substrates and/or one or more of the products of the following reactions in the test sample:

(*Ri*) acetyl phosphate + ADP $\xleftrightarrow{\text{acetate kinase}}$ acetyl phosphate + ATP

(*Rii*) carbamoyl phosphate + ADP $\xleftrightarrow{\text{carbamate kinase}}$ ammonia + ATP + carbonate

Advantageously, the method further comprises the steps of:

(c) obtaining detection and/or measurement information from a corresponding control sample;

(d) comparing the information from steps (b) and (c); and

(e) identifying the test sample as contaminated with mycoplasma if the detection and/or measurement information is different between the test and control samples.

Preferably, the detecting and/or measuring step comprises detecting and/or measuring ATP. Still more preferably, the ATP is detected and/or measured by a light-emitting reaction, especially a bioluminescent reaction.

Light-emitting systems have been known and isolated from many luminescent organisms, including certain bacteria, protozoa, coelenterates, molluscs, fish, millipedes, flies, fungi, worms, crustaceans, and beetles, particularly the fireflies of the genera *Photinus*, *Photuris*, and *Luciola* and click beetles of genus *pyrophorus*. In many of these organisms, enzymatically catalyzed oxidoreductions take place in which the free energy change is utilised to excite a molecule to a high energy state. Then, when the excited molecule spontaneously returns to the ground state, visible light is emitted. This emitted light is called "bioluminescence".

Beetle luciferases, particularly that from the firefly species, *Photinus pyralis*, have served as paradigms for understanding of bioluminescence since the earliest studies. The *P.pyralis* luciferase is an enzyme which appears to have no prosthetic groups or tightly bound metal ions and has 550 amino acids and a molecular weight of about 60,000 daltons; the enzyme has been available to the art in crystalline form for many years. Studies of the molecular components in the mechanism of firefly luciferases in producing bioluminescence have shown that the substrate of the enzymes is firefly luciferin, a polyheterocyclic organic acid, D-(-)-2-(6'-hydroxy-2'-benzothiazolyl)$\Delta^2$-thiazoline-4-carboxylic acid (herein-after referred to as "luciferin", unless otherwise indicated).

ATP can be detected using the following bioluminescent reaction.

$$\text{ATP} + \text{Luciferin} + O_2 \xrightarrow[Mg^{2+}]{\text{Luciferase}} \text{Oxyluciferin} + \text{AMP} + PP_i + CO_2 + \text{light (emission at 565 nm)}$$

The emitted light intensity is linearly related to the ATP concentration and is measured using a luminometer.

Luciferase has been used as a means of assaying minute concentrations of ATP; as little as $10^{-16}$ molar ATP can be detected with high quality preparations of the enzyme. The luciferase-luciferin reaction is highly specific for ATP. For example, deoxy-ATP produces less than 2% of the light generated by ATP, and other nucleoside triphosphates produce less than 0.1%.

Crystalline luciferases can be isolated directly from the light organs of beetles. cDNAS encoding luciferases of several beetle species (including, among others, the luciferase of *P.pyralis* (firefly), the four luciferase

isozymes of *P.plagiophthalamus* (click beetle), the luciferase of *L.cruciata* (firefly) and the luciferase of *L. lateralis*) (de Wet et al., 1987, Masuda et al., 1989, Wood et al., 1989, European Patent Application Publication No. 0 353 464) are available. Further, the cDNAs encoding luciferases of any other beetle species, which make luciferases, are readily obtainable by the skilled using known techniques (de Wet et al., 1986, Wood et al., 1989). With the cDNA encoding a beetle luciferase in hand, it is entirely straightforward to prepare large amounts of the luciferase in highly pure form by isolation from bacteria (e.g. *E.coli*), yeast, mammalian cells in culture, or the like, which have been transformed to express the cDNA.

Further, the availability of cDNAs encoding beetle luciferases and the ability to rapidly screen for cDNAs that encode enzymes which catalyze the luciferase-luciferin reaction (see de Wet et al., 1986, supra, and Wood et al., supra) also allow the skilled person to prepare, and obtain in large amounts in pure form, mutant luciferases that retain activity in catalyzing production of bioluminescence through the luciferase-luciferin reaction. Such a mutant luciferase will have an amino acid sequence that differs from the sequence of a naturally occurring beetle luciferase at one or more positions (White et al., 1996, WO 01/31028 and WO 00/24878). In the present disclosure, the term "luciferase" comprehends not only the luciferases that occur naturally in beetles but also the mutants, which retain activity in providing bioluminescence by catalyzing the luciferase-luciferin reaction, of such naturally occurring luciferases.

It is most preferred that in the method of the invention, between step (i) and (ii) and/or (a) and (b), the sample is treated so as to lyse any mycoplasma and thereby release their cellular contents into the sample. Skilled persons will understand that lysis can be effected by a variety of methods including application of chemicals, such as detergents and mechanical methods such as sonication etc.

Advantageously, the lysis is effected by treating the sample with a detergent, or other lysis method, which allows for the lysis of the Mycoplasma cell membrane but which does not affect the cell wall of any bacteria which may be present. Exemplary detergent treatment includes the use of low concentrations (e.g. 0.25% v/v) of a detergent, such as Triton X100.

The preferred lysis method is one that is sufficient to lyse the mycoplasmal membrane, but would be ineffective against bacterial cells. In studies comparing eukarytic cell lysis and bacterial lysis, it has been observed that non-ionic detergents (mainly polyethoxyethers) could be used to lyse somatic cells without affecting microbial cells (Schramm and Weyens-van Witzenberg 1989, Stanley 1989). It is the presence of the rigid cell wall that makes bacteria less sensitive to detergent lysis, and more rigorous lysis procedures are required to lyse bacterial cells. For efficient lysis and total protein release, bacteria often require exposure to enzymes such as lysozyme to breach the cell wall (Pellegrini *et al* 1992). The most preferred detergent mycoplasma lysis conditions are shown hereinafter.

However, a contamination with bacteria will produce turbid growth, and bacteria are also visible when viewing a cell culture under phase contrast microscopy. These bacterial cultures can be detected quite easily and discarded straight away.

Unlike bacteria, mycoplasma will pass through a 0.45μM filter used for filter sterilisation (Baseman and Tully 1997), and it is possible to distinguish between a bacterial and mycoplasmal contamination through the addition of a filtration step.

Hence, in preferred embodiments of the invention the test sample is passed through a bacterial filter in step (i) and (a). Of course, skilled persons will appreciate that if the test sample is treated to remove bacteria, for example by passing it through a bacteria-retaining filter, it is not important to lyse mycoplasma selectively, i.e. without lysing bacteria.

In a preferred embodiment, ADP is added to the test sample prior to the detecting and/or measuring step (ii) or (b). However, the assay can also utilise endogenous ADP.

In a preferred embodiment, a mycoplasma substrate reagent is added to the test sample prior to the detecting and/or measuring step (ii) or (b), the mycoplasma substrate reagent comprising: acetyl phosphate or a precursor thereof and/or carbamoyl phosphate or a precursor thereof.

By "a precursor thereof" we include one or more compounds from which acetyl phosphate and/or carbamoyl phosphate can be generated. Exemplary reactions are outlined below:

(i) acetyl–CoA $\xrightarrow{\text{phosphotransacetylase}}$ acetyl phosphate

(ii) citrulline + ammonia $\xrightarrow{\text{ornithine carbamoyl transferase}}$ carbamoyl phosphate + ornithine

Hence, instead of adding acetyl phosphate to the mycoplasma substrate reagent, one could include a precursor, such as acetyl-CoA.

Similarly, instead of carbamoyl phosphate one could add a precursor, such as citrulline and ammonia to the mycoplasma substrate reagent.

It is most preferred that both acetyl phosphate and carbamoyl phosphate and/or precursors thereof are added to the sample prior to step (ii), or (b),

in the methods of the invention. This enables a generic assay for mycoplasma contamination to be carried out because mycoplasmas utilise either or both substrates by means of their acetate kinase and/or carbamate kinase enzymes.

Alternatively, a more specific assay can be produced by only using one of the above substrates or precursors thereof. Such an assay will be specific for mycoplasma which only use one of the enzymes acetate kinase or carbamate kinase. The following table 2 cites some examples of the members of the Mollicutes family (parasitise mammalian hosts) that utilise acetate kinase preferentially, carbamate kinase preferentially, or both. In addition to those listed below there are a number of reptile, insect and plant infecting mycoplasmas where biochemical investigations have identified the use of these same pathways (Kirchoff *et al* 1997, Forsyth *et al* 1996, Taylor *et al* 1996 and Tully *et al* 1994).

**Table 2: ATP generation by mycoplasma through glucose or arginine utilisation.**

| Species | Preferential ATP Generation Pathway | Enzymes Utilised |
|---|---|---|
| *M. hyorhinis* | Glucose fermentation and Arginine lysis | Acetate kinase/Carbamate kinase |
| *M.orale* | Arginine lysis | Carbamate kinase |
| *M.fermentans* | Arginine lysis and glucose fermentation | Carbamate kinase /Acetate kinase |
| *M.salivarum* | Arginine lysis | Carbamate kinase |
| *M.arginii* | Arginine lysis | Carbamate |
| *A.laidlawii* | Glucose fermenting | Acetate kinase |
| *U.urealyticum* | Glucose fermenting | Acetate kinase |
| *M.pneumoniae* | Arginine lysis and glucose fermentation | Carbamate kinase /Acetate kinase |
| *M.mycoides* | Glucose fermenting | Acetate kinase |
| *M.arthritidis* | Arginine lysis | Carbamate kinase |
| *Anaeroplasma sp* | Arginine lysis | Carbamate kinase |
| *M.hominis* | Arginine lysis | Carbamate kinase |
| *A.vituli* | Glucose fermenting | Acetate kinase |
| *M.lagogenitalium* | Glucose fermenting | Acetate kinase |
| *M.mycoides* | Glucose fermenting | Acetate kinase |
| *M.penetrans* | Arginine lysis and glucose fermentation | Carbamate kinase/Acetate kinase |
| *M.pirum* | Arginine lysis and glucose fermentation | Carbamate kinase/Acetate kinase |
| *M.incognitis* | Arginine lysis and glucose fermentation | Carbamate kinase/Acetate kinase |

Most preferably, in all of the methods of all aspects of the invention, the "corresponding control sample" is the test sample prior to a mycoplasma lysis treatment and/or addition of a mycoplasma substrate and/or a time interval (e.g. more than approximately 30 minutes). In this preferred embodiment both of the activity measurements are carried out on the same sample, the test sample. A first activity measurement (A) is taken either before or concurrent with a mycoplasma lysis step then, after addition of a mycoplasma substrate and/or a time interval (e.g. more than approximately 30 minutes), a second activity measurement (B) is taken. If the value of $\frac{B}{A}$ is greater than one the test sample is identified as contaminated with mycoplasma.

Skilled persons will appreciate that the "corresponding control sample" could also be a predetermined negative control sample, but this is less preferred.

In an embodiment, the control sample has been shown to be free from mycoplasma contamination. Suitable methods for doing this include PCR testing, DNA fluorescent staining or culture methods as described herein. Thus, in one embodiment, by "corresponding control sample" we mean a sample which contains substantially the same material as that contained in the test sample, but which, unlike the test sample, has been shown to be free from mycoplasma contamination. Skilled persons will appreciate that a mycoplasma uncontaminated condition can be shown by a variety of known methods. A number of suitable methods are reviewed by Rottem and Barile 1993, while an outline of testing kits and services is given in Raab *et al* 1999.

The test sample can be a cell sample, such as a cell culture sample, especially a culture of mammalian cells. Some examples are listed in the following table 3.

**Table 3: Commonly cultured cell lines that have been tested using the assay method.**

| Cell Name | Cell type | Supplier/Deposit Number |
|---|---|---|
| K562 | Human Chronic Myelogenous Leukaemia | ECACC 89121407 |
| U937 | Human Histiocytic Lymphoma | ECACC 87010802 |
| HL-60 | Human Promyelocytic | ECACC 88112501 |
| Cem-7 | Human Acute T-Lymphoblastic Leukaemia | ATCC CCL-119 |
| Jurkat | Human T-Cell Leukaemia | ECACC 88042803 |
| CHO | Chinese Hamster Ovary | ECACC 85050302 |
| COS-7 | Simian Kidney Cells, SV40 transformed | ECACC 87021302 |
| Vero | African Green Monkey Kidney Cells | ECACC 84113001 |
| MRC5 | Human Foetal Lung | ECACC 84101801 |
| HUVEC | Human Umbilical Vein Endothelial Cells | ECACC 89110702 |
| BSMC | Human Broncial Smooth Muscle Cells | Cambrex CC-2576 |
| NHEK | Normal Human Epidermal Keratinocytes | Cambrex CC-2503 |
| MCF-7 | Human Breast Adenocarcinoma | ECACC 86012803 |
| AoSMC | Aortic Smooth Muscle Cells | Cambrex CC-2571 |
| A549 | Human Lung Carcinoma Cells | ECACC 86012804 |
| HepG2 | Human Hepatocyte Carcinoma | ECACC 85011430 |
| FM3A | Mouse Mammary Carcinoma | ECACC 87100804 |
| PC12 | Rat Adrenal Pheochromocytoma | ECACC 88022401 |
| ARPE-19 | Human Retinal Pigment Epithelial Cells | ATCC CRL-2302 |
| RT112 | Human Bladder Carcinoma | ECACC 85061106 |

*Where ECACC represents the European Collection of Animal Cell Culture, ATCC represents the American Tissue Culture Collection, and Cambrex represents Cambrex Bio Science Wokingham, UK*

Also, it would be possible to test mammalian primary cell types, plus all those cells held by tissue banks, for example the ATCC and ECACC.

It is notable that the assays of the invention can be utilised to detect mycoplasma contamination in cultures of both adherent cells (e.g. HepG2, A549, CHO and COS cells) and cells which culture in suspension (e.g. Jurkats, U937, K562 and HL-60 Cells.)

Preferably the sample to be tested is from the cell culture supernatant which has previously been centrifuged to remove cellular material. However, it is also possible to perform the assay in the presence of cells or cellular debris.

Cell-free samples can also be tested using the methods of the invention. For example, the methods of the invention are particularly useful for testing samples of cell-free reagents, such as tissue culture media, and typically those containing animal-derived materials, such as serum (e.g. foetal calf serum), trypsin, and other culture supplements, etc. Examples of some commonly used media and supplements that may be tested in this manner are shown in table 4.

**Table 4: Tissue culture media and supplements that may be tested using the assay system.**

| Culture Media | Sera | Growth Factors | Other Tissue Culture Reagents |
|---|---|---|---|
| RPMI | Foetal Calf | Epidermal growth factor | Trypsin |
| DMEM | Newborn Calf | Transforming growth factor | Insulin |
| Eagle's MEM | Horse | Granulocyte-colony stimulating factor | Transferrin |
| Glasgow MEM | Human | Granulocyte-macrophage CSF | Collagen |
| Ham's F12 | Porcine | Nerve growth factor | Fibronectin |
| IMDM | Chicken | | Vitronectin |
| Medium 199 | Rabbit | | Amino acid supplemnts |
| McCoy's 5A | Sheep | | Gelatin |
| Hybridoma | | | Albumins |
| CHO media | | | Pancreatin |
| Embryo Culture Media | | | Bovine pituitary extract |
| Williams Medium E | | | |

A third aspect of the invention provides a method of detecting the presence of mycoplasma in a test sample, comprising the following steps:-

(i) providing a test sample;

(ii) without adding an exogenous reagent (e.g. substrates for kinase activity) to convert ADP to ATP, detecting or measuring ATP in the test sample using a bioluminescent reaction to obtain an ATP and/or light output measurement (A);

(iii) obtaining an ATP and/or light output measurement (B) from a corresponding control sample;

(iv) comparing the ATP and/or light output measurement ratio $\frac{A}{B}$; and

(v) identifying the test sample as contaminated with mycoplasma in the event that the ratio $\frac{A}{B}$ is greater than one.

As mentioned in connection with the earlier aspects of the invention, it is most preferred that Mycoplasma lysis treatment and/or addition of mycoplasma substrate and/or a time interval occurs before step (ii).

As mentioned in connection with the earlier aspects of the invention, it is most preferred that the "corresponding control sample" is the test sample except that it has not been subjected to Mycoplasma lysis treatment and/or addition of mycoplasma substrate and/or left for a time interval (e.g. more than approximately 30 minutes).

In other words, both measurements are taken from the test sample. Thus, in a preferred embodiment, the control ATP and/or light output measurement is taken following addition to the sample of the mycoplasma detection reagent containing the detergent and liciferase/luciferin plus AMP, and the test ATP and/or light output measurement is taken following addition of

substrates for kinase activity (or precursors thereof).

A fourth aspect of the invention provides an *in vitro* process for treating a cell culture to remove mycoplasma contamination comprising:- treating a mycoplasma contaminated cell culture with an agent to remove or destroy mycoplasma; and subsequently testing a sample from the culture for mycoplasma contamination using a method of the invention; if necessary, repeating the process one or more times until mycoplasma contamination is not detected in the sample.

Most routine antibiotics used in cell culture are ineffective against mycoplasma. There are some agents that show inhibitory activity, these include gentamicin sulfate, kanamycin sulfate and tylosin tartrate (www.unc.edu/depts/tcf/mycoplasma.htm). There are a number of commercial treatment products, including Mycoplasma Removal Agent (ICN-Flow), a derivative of the quinolone family of antibiotics, also a non-antibiotic treatment from Minerva Biolabs (Berlin, Germany), Mynox®. The US company Invivogen supply Plasmocin™, which has two bactericidal components, one that acts on protein synthesis and the other that inhibits DNA replication. The antibiotics tetracycline and ciprofloxacin are reported to have success rates of less than 80-85% (www.unc.edu/depts/tcf/mycoplasma.htm). It is therefore extremely difficult to completely irradicate mycoplasma from cultures, once a contamination has taken hold.

Most of the effective antibiotics are quinolone derivatives, and the effectiveness of different antibiotics varies according to the mycoplasma species being tested. Duffy *et al* 2000, investigated *M.pneumoniae, M.hominis, M.fermentans, M.genitalium* and *U.urealyticum* viability against the quinolone gemifloxacin, and compared with a number of antibiotics

including tetracycline, clindamycin and other quinolones. The results showed variable responses between species, however gemifloxacin performed better than tetracycline. There are some species that show resistance to tetracyclines, due to acquisition of the tetM gene. This is a frequent occurrence, and is complicated by variations in the responses of species dependent upon the source of mycoplasma. For example, mycoplasma exposed to antibiotics in eukaryotic cell culture have different profiles from the same species isolated from a human or animal source (Taylor-Robinson and Bebear 1997). While the reported success of anti-mycoplasma treatments appears highly variable, a recent study by Uphoff *et al* 2002, reports that 96% of leukaemia-lymphoma cell lines were rendered free of mycoplasma with at least one of the treatments tested.

Examples which embody various aspects of the invention will now be described with reference to the accompanying figures in which:-

**Figure 1:** The kinetics of ATP generation in the presence of *M.hyorhinis* contamination.

**Figure 2:** A Comparison between the PCR kit from Stratagene and a preferred embodiment of the invention ratios.

**Figure 3:** Treatment of contaminated cell lines with Mycoplasma Removal Agent according to a preferred embodiment of the invention.

**Figure 4:** Ratio data with cells, supernatants and supernatants filtered through a 0.45μm (F1), 0.22μm (F2) and 0.1μm (F3) filters.

**Figure 5:** Effect of supernatant dilution.

**Figure 6:** *M. fermentans* at 7900 CFUs/well, tested against the different substrates.

**Figure 7:** *M. orale* stock at 1450 CFUs/well, tested against the different substrates.

**Figure 8:** Dilution of the *M.orale* stock to show sensitivity of the assay.

**Figure 9:** *M. hyorhinis*, comparison of different substrate reagents.

**Figure 10:** The effect of Triton-X100 concentrations on the detection of mycoplasma enzyme activities in K562 cells infected with *M.hyorhinis* (MH) and *M.orale* (MO).

**Figure 11:** Shows the effect of increasing Triton-X100 concentrations on K562 cells contaminated with M.hyorhinis (MH) compared to increasing numbers (1-10,000 cells/100µl sample) of bacterial cells (E.coli).

**Example 1: Assay method of the invention**

The principle of the preferred assay method is to supply the appropriate substrates for mycoplasmal enzymes. If mycoplasma contamination is present, there is a conversion of ADP to ATP which can then be measured, preferably by the luciferase-luciferin reaction.

Mycoplasma Detection Reagent is added and, after approximately 5 minutes, an initial light output reading (A) is taken, the Mycoplasma Substrate (MS) is added and any enzymatic activity is allowed to progress for approximately 10 minutes, at which point a second light reading (B) is taken.

If there is mycoplasmal contamination then the second reading (B) will be higher when compared to the first reading (A), giving a ratio $\frac{B}{A}$ of greater than 1. If the culture is negative (uncontaminated by mycoplasma), then the ratio $\frac{B}{A}$ will be 1 or most often less than one due to the luminescent light signal decay usually seen over time. Figure 1 demonstrates the kinetics of the reaction. Typically, the ratio $\frac{B}{A}$ seen with mycoplasma contamination is much greater than 1, for example Figure 1 shows a ratio of 114.

A preferred assay kit of the invention comprises a Mycoplasma Detection Reagent (MDR); Mycoplasma Assay Buffer (MAB) for reconstitution of MDR and the Mycoplasma Substrate (MS). MDR and MS are preferably provided as lyophilised preparations.

All mycoplasma generate ATP through either the acetate kinase pathway or the carbamate kinase pathway. The Mycoplasma Substrate of the invention contains substrates for one or both of these enzymatic reactions. ADP is a requirement for both enzymes, and is preferably supplied in excess in the Mycoplasma Detection Reagent of the invention to drive the generation of ATP formation.

The MDR is added to a sample of culture supernatant that has previously been centrifuged to remove cellular material, although it is possible to perform the assay in the presence of cells. Alternatively or additionally, the test sample can be passed through a bacterial filter.

The MDR contains substrates for luciferase, luciferin and other co-factors plus AMP and ADP. The Mycoplasma Substrate (MS) contains carbamoyl phosphate and/or acetyl phosphate or precursors thereof, required for detection of the carbamate and/or acetate kinase activities.

A preferred sample volume is 100µl to which 100µl of reconstituted MDR is added. After approximately 5 minutes the first luminometric reading (A) is taken, this gives the base reading upon which the further ratio calculations $\frac{B}{A}$ are determined.

The assay methods of the invention have been used to investigate contamination by *Acholeplasma laidlawii, M. hyorhinis, M. fermentans, M. orale,* and *M. genitalium* and to detect a number of unknown mycoplasma contaminations.

The inventors have compared their data to detection of mycoplasma by PCR, and have shown that there is a correlation between ratios greater than one and detection of mycoplasmal DNA. This is shown in Figure 2 where the positive PCR bands on the gel correlate with ratios of more than one.

**Example 2: use of the methods of the invention in a process for removing mycoplasma contamination from a cell culture.**

The inventors have also shown that we can detect a reduction in ratios $\frac{B}{A}$ as cells are treated with an exemplary Mycoplasma Removal Agent (ICN-Flow), a derivative of the quinolone family of antibiotics.

The manufacturers (ICN-Flow) recommend treatment for 7 days of cells in quarantine to ensure complete removal of contaminating mycoplasma. However, the ratio data obtained using the assay methods of the invention showed that 7 days was not sufficient. This was evident from the fact that the ratios remained greater than one. Also after removal of treatment, and continued culture the ratios increased, and the cultures were again positive after PCR testing (Stratagene kit). These data are shown in Figure 3, where three different cell lines were found to be contaminated with *M.hyorhinis*.

While the K562 and U937 cells are suspension cell lines, the A549 cells are an adherent cell type; these data therefore confirms that the assay can be used on both adherent and suspension cell types. This is also shown in Figure 2 where the CHO and COS-7 cells are adherent cell types commonly used in cell culture laboratories.

Figure 3 also shows that the treatment with MRA for 10 days with COS-7 and CHO cell cultures was sufficient to remove the contaminating mycoplasma.

**Example 3: Failure of bacterial filters to exclude mycoplasma**

The inventors have also shown that culture supernatants put through a number of bacterial retarding filters continued to show positive ratios which is indicative of the presence of viable mycoplasma. This is shown in Figure 4.

Mycoplasma can form colonies as large as 600μm in diameter, but can also exist in their life cycle as single cells as small as 0.15μm. Due to their small size mycoplasma can pass through the 0.45μm and 0.22μm filters commonly used to "sterilise" tissue culture reagents. Figure 4 also confirms that the assay can be performed in the presence of cells, but that there is a reduced sensitivity of detection. Hence, it is preferred that the assay methods of the invention are performed on samples which are substantially cell free. This can easily be achieved by centrifugation of cell cultures and sampling of the supernatant and, optionally, filtration through a bacterial filter.

**Example 4: Sensitivity of preferred assays**

As shown in Figures 5 and 8, dilution of the supernatants shows the sensitivity of the assays, in that a 1:1000 dilution of contaminated culture supernatant can still give ratios greater than 1. Dependent on the specific activity of the acetate kinase and carbamate enzymes in different mycoplasma (Mollicutes) species, it is possible to dilute samples out further. The dilution range will also vary according to the number of colony forming units in the test sample.

**Example 5: variations of the assay methods of the invention**

The assay methods of the invention will work without the external exogenous addition of carbamate and acetate kinase substrates in the form of ADP, carbamoyl phosphate and acetyl phosphate or precursors thereof. In a contaminated culture sample the acetyl and carbamoyl phosphates or precursors thereof will be present endogenously together with sufficient cellular ADP, derived from the cell culture, to prime the reaction towards the formation of ATP. Alternatively, ADP can be generated by other externally added or cellular enzymes i.e. adenylate kinase utilising ATP and AMP.

It is possible to avoid direct addition of these substrates and have the system generate them itself. The use of acetate and ammonia along with ATP will cause the acetate kinase and carbamate kinase enzymes to generate acetyl phosphate and carbamoyl phosphate that can then be used by the same enzymes to generate ATP from ADP:

Acetate + ATP <---(acetate kinase)---> Acetyl phosphate + ADP

Ammonia + ATP + carbonate <---(carbamate kinase)---> Carbamoyl phosphate + ADP

The two substrates could also be generated from "precursors" by utilising earlier parts of the glucose fermentation and argenine lysis pathways for example by the addition of acetyl-CoA and citrulline that could be used by mycoplasmal enzymes to synthesise acetyl phosphate and carbamoyl phosphate respectively.

acetyl-CoA ---(phosphotransacetylase)---> acetyl phosphate

citrulline + ammonia ---(ornithine carbamoyl transferase)---> carbamoyl phosphate + ornithine

The following figures show the differences between the biochemical activities of *M.fermentans*, which generates ATP preferentially through the carbamate kinase pathway, but will also utilise the acetate kinase pathway. Figure 6 shows the effect of adding the substrates for the enzyme pathways individually, and then in a combined reagent.

While *M.fermentans* utilises both pathways, *M.orale* utilises only the carbamate kinase pathway, and as shown in Figure 7, positive ratios are only observed in the single carbamate reagent or the combined reagent. Figure 8 shows the detection limits are as low as 14 CFU/well with *M.orale*.

The inventors have investigated a mycoplasma that preferentially utilises the acetate kinase pathway, namely *M.hyorhinis*. The data is shown in Figure 9.

The inventors have tested over 15 different cell lines (see table 3) and shown that none of the cells have sufficient background enzymatic activity to impact upon the ratios and give false positives. The inventors, without wishing to be bound by theory, think the reason for this is that the pathways are anaerobic, and all mammalian cell cultures will generate ATP through *oxidative* phosphorylation. Hence, by using only carbamoyl phosphate or a precursor thereof or only acetyl phosphate or a precursor thereof, one can produce an assay method of the invention which will allow one to determine whether the mycoplasmal contaminants in question are from a group which uses the acetate kinase pathway, the carbamate kinase pathway, or both. This may have useful diagnostic applications.

The only bacteria that have acetate kinase activity are not those that are commonly found as contaminants of cell culture, with the possible exception of certain *E.coli* species that are handled in laboratories, principally for molecular biology purposes. However, activity with this organism, is only seen at very high inoculum concentrations where there is turbid growth and the resulting turbidity of the sample is readily observed by eye. Hence, the methods of the invention can be varied to include an initial screening step for bacterial contamination, if necessary. This can be achieved by a variety of methods, but is preferably carried out by passing the sample through a standard bacterial filter (Baseman and Tully, 1997).

**Example 6: Preferred Reagent Components for use in the mycoplasma assay methods and kits of the invention**

1. Mycoplasma Detection Reagent (MDR) per 100ml

- Magnesium acetate 214.5mg (10mM)
- Inorganic pyrophosphate 178.4µg (4µM)

- Bovine serum albumin 160mg (0.16%)
- D-Luciferin 10mg (360µM)
- L-Luciferin 250µg (8.9µM)
- Luciferase (RY) 85µg
- ADP 250.5µg (5µM)
- AMP 69.44mg (2mM)

*RY is the name given to the recombinant luciferase supplied by Lucigen. A mixture of D and L-Luciferin has been found to give a more stable light output than D-luciferin alone.

2. Mycoplasma Substrate (MS) per 100ml

- Acetyl phosphate 55.23mg (3mM)
- Carbamoyl phosphate 45.87mg (3mM)

*Mycoplasma Substrate (Ms) Precursors*

Examples of reactions generating acetyl or carbamoyl phosphate:

i. acetyl-CoA + inorganic phosphate —*phosphotransacetylase*→ acetyl phosphate + CoA

Preferred concentration ranges:

| | |
|---|---|
| acetyl-CoA | 0.1mM to 100mM |
| Inorganic phosphate (e.g. potassium phosphate) | 0.1mM to 100mM |

ii. citrulline + ammonium bicarbonate —*ornithine carbamoyl transferase*→ carbamoyl phosphate + ornithine

Preferred concentration ranges:

| | |
|---|---|
| citrulline | 1mM to 100mM |
| ammonium bicarbonate | 1mM to 200mM |

iii. (sodium or potassium) acetate + ATP $\xrightarrow{\textit{acetate kinase}}$ acetyl phosphate + ADP

Preferred concentration ranges:

| | |
|---|---|
| acetate (e.g. sodium or potassium) | 1mM to 500mM |
| ATP | 0.1mM to100mM |

iv. ATP + ammonium bicarbonate $\xrightarrow{\textit{carbamate kinase}}$ carbamoyl phosphate + ADP

Preferred concentration ranges:

| | |
|---|---|
| ammonium bicarbonate | 1mM to 200mM |
| ATP | 0.1mM to 100mM |

**Suppliers**

Sigma-Aldrich Company Ltd.
Fancy Road
Poole
Dorset
BH12 4QH
United Kingdom

3. Mycoplasma Assay Buffer (MAB) per 100ml

| | |
|---|---|
| • HEPES | 1.1915g (50mM) |
| • EDTA | 74.44mg (2mM) |
| • Triton X-100 | 250µl (0.25%) |
| • pH 7.50 | |

**Preferred concentration ranges of Components for use in the Mycoplasma assay methods and kits of the invention**

Preferred concentration ranges include

- ADP 1μM to 100mM, preferably 1 to 100μM, more preferably 5μM.
- AMP 1μM to 100mM, preferably 0.1mM to 10mM, more preferably 2mM.
- Acetyl phosphate 1μM to 100mM, preferably 0.1mM to 20mM, more preferably 3mM. Concentrations above 10 reduce the light output, but the assay still performs.
- Carbamoyl phosphate 1μM to 100mM, preferably 0.1mM to 20mM, more preferably 3mM.

**Example 7: Effects of Detergents on Mycoplasma Assay**

Disruption of the viable mycoplasma cell membrane to allow for the release of the enzymes into the sample is a preferred embodiment of the assay method of the invention. This allows for binding of the substrates and generation of ATP. However, positive ratios indicating mycoplasma contamination can be obtained in the absence of any lysis treatment. The implication is that these enzymes can be released by viable mycoplasma The other possibility is that some non-viable organisms have released their contents through natural lysis.

The addition of even low concentrations of the non-ionic detergent Triton-X100, greatly increases the sensitivity of the assay by ensuring maximal release of the carbamate and acetate kinases into the sample.

The aim of the following experiments was to determine the concentrations of Triton-X100 in the Hepes-EDTA buffer on the ratios seen with

mycoplasma contaminated K562 cell cultures. Two organisms were investigated, *M.hyorhinis* and *M.orale*.

**Figure 10** shows that it is possible to detect mycoplasmal enzymes in the absence of a detergent lysis step. It also shows a drop in the light output with concentrations greater than 4-5%, this is due to adverse effects of the detergent on the luciferase enzyme/reaction. However, it is still possible to detect positive activity with concentrations as high as 20% (v/v).

The inventors have also shown that the concentrations of Triton-X100 used in the above experiments did not result in any detectable carbamate or acetate kinase activity from the *E.coli* strain JM109 cells.

The above results confirm that there are no positive ratios with the bacterial cell number used. Increasing the Triton-X100 concentration to levels that have been reported to lyse bacterial cells (1-2%), still did not result in positive ratios above 1.

Generally biological detergents are commonly used to disrupt the bipolar membranes of lipids in order to release and then solubilise membrane bound proteins. Non-ionic detergents are non-denaturing and permit the solubilization of membranes without interfering with biological activity. They have principally been used for the study of protein conformations and for the separation of hydrophilic proteins from membrane spanning hydrophobic proteins. Anionic and cationic detergents result in greater modification of protein structure and are more effective at disrupting protein aggregation. Zwitterionic detergents are also low-denaturing, but are effective at disruption of protein aggregates.

These different groups of detergents have been studied with a number of

different cells types to efficiently lyse, and release and preserve the protein content, of both eukaryotic and prokaryotic organisms.

For the preferred Assays of the invention the required lysis agent is one that causes disruption of the mycoplasmal membrane and allows release of the metabolic enzymes that are required to react with the substrates. As there is no detergent removal or neutralisation step, it is therefore important that the chosen system does not interfere with the activity of the carbamate and/or acetate kinase, or the luciferase/luciferin/ATP reaction. It is also preferable to use a system that selectively causes the lysis of mycoplasma, with little or no effect on bacteria that may be potential contaminates of the cell cultures/samples.

The presence of a filtration step through 0.45µm filters, however, should remove any contaminating larger micro-organisms.

The key difference between bacteria and mycoplasma is the lack of cell wall, and it is the bacterial cell wall that makes bacteria more difficult to lyse. There are a number of fairly brutal methods that can bring about total lysis, these include pressure (French Press) and sonication. Other enzyme digest methods include lysozyme followed by the addition of detergents. However, mycoplasma can be lysed with concentrations of Triton X-100 at around 1-2%.

Low concentrations of other non-ionic detergents, such as Brij®35 (0.4%) (Sigma-Aldrich Company Ltd.) and B-PER (1%) (Perbio Science UK Ltd.), do not have adverse effects on the luciferase enzyme, and are capable of disrupting the mycoplasmal membrane, without adversely affecting the luciferase reaction. The concentrations of these detergents can be taken up to 10% without loss of sensitivity of mycoplasmal detection.

Contaminating mycoplasma can be detected in the absence of a lysis step to disrupt the mycoplasmal membrane. However, addition of a gentle lysis step (0.25% Triton X-100 in Hepes-EDTA buffer) increases the sensitivity of the assay by releasing the mycoplasmal enzymes of interest into the reaction mixture.

The lysis step would preferably cause selective lysis of mycoplasma, while having little or no effect on bacterial cells. Low concentrations of most non-ionic detergents should do this. However, a filtration step would physically remove any contaminating bacteria, and allow for the use of any detergent but preferably those that do not inhibit either the luciferase reaction or the activity of carbamate kinase and acetate kinase.

**Example 8: preferred kit contents**

**LT07-118** (Sufficient for 10 tests)

1. LT27-217 Mycoplasma Detection Reagent, Lyophilised. 2 x 600 µl vials.
2. LT27-218 Mycoplasma Assay Buffer. 1 x 10 ml bottle.
3. LT27-221 Mycoplasma Substrate. Lyophilised. 2 x 600 µl vials.

**LT07-218** (Sufficient for 25 tests)

1. LT27-217 Mycoplasma Detection Reagent. Lyophilised. 5 x 600 µl vials.
2. LT27-218 Mycoplasma Assay Buffer. 1 x 10 ml bottle.
3. LT27-221 Mycoplasma Substrate. Lyophilised. 5 x 600 µl vials.

LT07-318 (Sufficient for 100 tests)

1. LT27-216 Mycoplasma Detection Reagent. Lyophilised. 1 x 10 ml vial.
2. LT27-220 Mycoplasma Assay Buffer. 1 x 20 ml bottle.
3. LT27-224 Mycoplasma Substrate. Lyophilised. 1 x 10 ml vial.

**Preferred reagent compositions for kits and methods of the invention**

1. Mycoplasma Detection Reagent (MDR) per 100ml

| | |
|---|---|
| • Magnesium acetate[1] | 214.5mg (10mM) |
| • Inorganic pyrophosphate[1] | 178.4µg (4µM) |
| • Bovine serum albumin[1] | 160mg (0.16%) |
| • D-Luciferin[2] | 10mg (360µM) |
| • L-Luciferin[2] | 250µg (8.9µM) |
| • Luciferase (RY)[3] | 85µg |
| • ADP[1] | 250.5µg (5µM) |
| • AMP[1] | 69.44mg (2mM) |

2. Mycoplasma Substrate (MS) per 100ml

| | |
|---|---|
| • Acetyl phosphate[1] | 55.23mg (3mM) |
| • Carbamoyl phosphate[1] | 45.87mg (3mM) |

3. Mycoplasma Assay Buffer (MAB) per 100ml

| | |
|---|---|
| • HEPES[1] | 1.1915g (50mM) |
| • EDTA[1] | 74.44mg (2mM) |
| • Triton X-100[1] | 250µl (0.25%) |
| • pH 7.50 | |

Preferred concentration ranges:

- ADP 1µM to 100mM
- AMP 1µM to 100mM
- Acetyl phosphate 1µM to 100mM, preferably, mM to 10mM

- Carbamoyl phosphate 1μM to 100mM

**Suppliers**

1.
Sigma-Aldrich Company Ltd.
Fancy Road
Poole
Dorset
BH12 4QH
United Kingdom

2.
Resem BV
Goudenregenstraat 84
NL-4131 BE Vanen
Netherlands

3.
Lucigen Ltd
Porton Down Science Park
Porton, Salisbury
Wiltshire SP4 0JQ
U.K.

The preferred embodiment of the invention provides a selective biochemical test that exploits the activity of certain mycoplasmal enzymes. The presence of these enzymes provides a rapid screening procedure, allowing sensitive detection of contaminating mycoplasma in a test sample. The viable mycoplasma are lysed and the enzymes react with the Mycoplasma Substrate catalysing the conversation of ADP to ATP.

By measuring the level of ATP in a sample both before (A) and after (B) the addition of the Mycoplasma Substrate, a ratio $\frac{B}{A}$ can be obtained which is

indicative of the presence or absence of mycoplasma. If these enzymes are not present, the second reading shows no increase over the first (A), while reaction of mycoplasmal enzymes with their specific substrates in the Mycoplasma Substrate Reagent, leads to elevated ATP levels. This increase in ATP can be detected using the following bioluminescent reaction.

$$\text{ATP} + \text{Luciferin} + O_2 \xrightarrow[Mg^{++}]{\text{Luciferase}} \text{Oxyluciferin} + \text{ADP} + PP_i + CO_2 + \text{light (emission at 565nm)}$$

The emitted light intensity is linearly related to the ATP concentration and is measured using a luminometer. The assay is preferably conducted at ambient room temperature (18-22°C), the optimal temperature for luciferase activity.

**Simple test protocol of the invention**

Add detection reagent (MDR) to sample

↓

Wait (e.g. 5 mins)

↓

Measure luminescence (Reading A)

↓

Add mycoplasma substrate (MS) to sample

↓

Wait (e.g. 10 mins)

↓

Measure luminescence (Reading B)

If $\frac{B}{A}$ is greater than one = mycoplasma contaminated sample.

If $\frac{B}{A}$ is one or less = mycoplasma free sample.

**Outline of the method**

It is preferred that the culture supernatant be centrifuged to remove cells and, optionally, passed through a bacterial filter prior to performing the assay.

The kit contains all the required reagents to perform the assay.

100µl of culture supernatant is taken as the sample.

Add Mycoplasma detection reagent (MDR)

Wait 5 minutes

Read luminescence (A)

Add Mycoplasma Substrate (MS)

Wait 10 minutes.

Read luminescence (B).

*Reagent reconstitution and storage*

Ensure that you follow the correct reagent reconstitution applicable to the relevant kit (10, 25 or 100 assay points).

This procedure usually requires at least 15 minutes equilibration time.

The Mycoplasma Detection Reagent (MDR) and Mycoplasma Substrate (MS) are preferably supplied as lyophilised pellets. These are reconstituted in Mycoplasma Assay Buffer (MAB) to produce the working solutions for use in the assay.

For 10 tests (KIT LT07-118):

I. Preparation of Mycoplasma Detection Reagent

Add 600µl of Mycoplasma Assay Buffer into a vial containing the lyophilised Mycoplasma Detection Reagent.

Replace the cap and mix gently.

Allow the reagent to equilibrate for 15 minutes at room temperature.

2. Preparation of Mycoplasma Substrate

Add 600µl of Mycoplasma Assay Buffer into a vial containing the lyophilised Mycoplasma Substrate.

Replace the cap and mix gently.

Allow the reagent to equilibrate for 15 minutes at room temperature.

3. Mycoplasma Assay Buffer

This is preferably provided ready for use. Store at 2-8°C when not in use.

For 25 tests (KIT LT07-218)

1. Preparation of Mycoplasma Detection Reagent

Add 600µl of Mycoplasma Assay Buffer into a vial containing the lyophilised Mycoplasma Detection Reagent.

Replace the cap and mix gently.

Allow the reagent to equilibrate for 15 minutes at room temperature.

2. Preparation of Mycoplasma Substrate

Add 600µl of Mycoplasma Assay Buffer into a vial containing the lyophilised Mycoplasma Substrate.

Replace the cap and mix gently.

Allow the reagent to equilibrate for 15 minutes at room temperature.

3. Mycoplasma Assay Buffer

This is preferably provided ready for use. Store at 2-8 when not in use.

For 100 tests (KIT LT07-318)

1. Preparation of Mycoplasma Detection Reagent

Add 10ml of Mycoplasma Assay Buffer into a vial containing the lyophilised Mycoplasma Detection Reagent.

Replace the cap and mix gently.

Allow the reagent to equilibrate for 15 minutes at room temperature.

2. Preparation of Mycoplasma Substrate

Add 600µl of Mycoplasma Assay Buffer into a vial containing the lyophilised Mycoplasma Substrate.

Replace the cap and mix gently.

Allow the reagent to equilibrate for 15 minutes at room temperature.

3. Mycoplasma Assay Buffer

This is preferably provided ready for use. Store at 2-8 when not in use.

*Equipment*

1. Instrumentation

The kit requires the use of a luminometer. The parameters of the luminometer should be assessed and the conditions below used to produce the correct programming of the machine.

The preferred assay of the invention has been designed for use with cuvette/tube luminometers. For use with plate luminometers please see below.

Cuvette/tube luminometers:

Read time 1 second (integrated).

2. Additional equipment and consumables

a. 10 ml sterile pipettes

b. Luminometer cuvettes

c. Micropipettes – 50-200µl; 200-1000 µl

d. Bench centrifuge.

Preferred test protocol

Please note samples of the culture medium should be taken before any further processing steps, e.g. trypsinisation.

1. Bring all reagents up to room temperature before use.
2. Reconstitute the Mycoplasma Detection Reagent and Mycoplasma Substrate in Mycoplasma Assay Buffer. Leave for 15 minutes at room temperature to ensure complete rehydration.
3. Transfer 2 ml of cell culture or culture supernatant into a centrifuge tube and pellet any cells at 1500 rpm (200 x g) for 5 minutes.
4. Transfer 100µl of cleared supernatant into a luminometer cuvette/tube.
5. Program the luminometer to take a 1 second integrated reading (this is usually the default setting on most cuvette luminometers).
6. Add 100µl of Mycoplasma Detection Reagent to each sample and wait 5 minutes.
7. Place cuvette in luminometer and initiate the programme (Reading A).
8. Add 100µl of Mycoplasma Substrate to each sample and wait 10 minutes.
9. Place cuvette in luminometer and initiate the programme (Reading B).
10. Calculate ration = Reading B/Reading A.

*Interpretation of results*

The ratio of Reading B to Reading A is used to determine whether a cell culture is contaminated by mycoplasma.

The speed and convenience offered by the kits according to the invention means that it provides a unique method for screening cultures for the presence of mycoplasma. As such it is ideally suited to routine testing of cells in culture. Frequent use of the test methods of the invention will indicate when a cell line becomes infected allowing prompt remedial action to be taken. The test methods of the invention can also be extended to incoming cell lines and the commonly used constituents of complete media.

The interpretation of the different ratios obtained, within each experimental situation, may vary according to the cell types and conditions used. However, the test gives ratios $\frac{B}{A}$ of less than 1 with uninfected cultures. Cells which are infected with mycoplasma will routinely produce ratios greater than 1.

**Table a. Interpretation of assay results: illustrating examples of healthy and infected cell lines.**

| Cell Line | Mycoplasma ratio | Conclusions |
|---|---|---|
| Infected cells | | |
| K562 | 123.26 | Positive |
| A549 | 4.10 | Positive |
| U937 | 8.26 | Positive |
| HepG2 | 1.27* | Borderline, quarantine and retest in 24 hours |
| Healthy cells | | |
| HL60 | 0.72 | Negative |
| COS-7 | 0.46 | Negative |

*Protocol for plate luminometers*

1. Bring all reagents up to room temperature before use.
2. Reconstitute the Mycoplasma Detection Reagent and Mycoplasma Substrate in Mycoplasma Assay Buffer. Leave for 15 minutes at room temperature to ensure complete rehydration.
3. Transfer 2 ml of cell culture of cell culture supernatant into a centrifuge tube and pellet any cells at 1500rpm (200 x g) for 5 minutes.
4. Transfer 100µl of cleared supernatant into a luminescence compatible plate.
5. Program the luminometer to take a 1 second integrated reading.
6. Add 100µl of Mycoplasma Detection Reagent to each sample and wait 5 minutes.
7. Place plate in luminometer and initiate the programme (ReadingA).

8. Add 100µl of Mycoplasma Substrate to each sample and wait 10 minutes.
9. Place plate in luminometer and initiate the programme (Reading B).
10. Calculate ratio = Reading B/Reading A.

Great care should be taken when handling any of the reagents. Skin has high levels of ATP on its surface that can contaminate the reagents leading to falsely high readings. Latex gloves avoid this problem.

The optimal working temperature for all reagents is 22°C. If reagents have been refrigerated always allow time for them to reach room temperature (18-22°C) before use.

The sensitivity of the assay does allow for detection of covert contamination, and if the ratio is marginally above 1 (for example up to 1.3) it is recommended that the sample be retested. Any cultures maintained in quarantine can be tested after a further 24-48 hours in culture to see if the ratios have increased.

**Summary**

The assays of the invention can be performed in the presence or absence of cells. Unlike known mycoplasma detection systems, they allow for samples to be screened rapidly using cheap hand-held luminometer systems, and can give results within 15 minutes to allow for the appropriate handling of the contaminated samples.

PCR and DAPI/Hoechst staining, will bind to all DNA, be it from viable or non viable mycoplasma. Hence, if looking to treat and remove mycoplasma, you could still end up with false positives when using PCR/DNA staining even though mycoplasma have been irradicated.

The assays can detect viable mycoplasma whereas known methods such as PCR cannot distinguish between viable and non-viable mycoplasma.

5

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## CLAIMS

1. A method of detecting the presence of mycoplasma in a test sample comprising:

(i) providing a test sample; and

(ii) detecting and/or measuring the activity (B) of acetate kinase and/or carbamate kinase in the test sample, said activity being indicative of mycoplasma contamination.

2. A method according to Claim 1 further comprising:

(iii) obtaining acetate kinase and/or carbamate kinase activity information (A) detected and/or measured in a corresponding control sample;

(iv) comparing the activity detected and/or measured in the test sample (B) with that in the control sample (A); and

(v) identifying the test sample as contaminated with mycoplasma if the activity (B) detected and/or measured in the test sample is greater than that of the control sample (A), that is, the ratio $\frac{B}{A}$ is greater than one.

3. A method as claimed in Claim 1 comprising:

(a) providing a test sample; and

(b) obtaining activity information by detecting and/or measuring the appearance and/or disappearance of one or more of the substrates and/or one or more of the products of the following reactions in the test sample:

(*Ri*) acetyl phosphate + ADP $\overset{\text{acetate kinase}}{\longleftrightarrow}$ acetate + ATP

(*Rii*) carbamoyl phosphate + ADP $\overset{\text{carbamate kinase}}{\longleftrightarrow}$ ammonia + carbonate + ATP.

4. A method according to Claim 3 further comprising:

(c) obtaining detection and/or measurement information (A) from a corresponding control sample;

(d) comparing the information (A) and (B) from steps (b) and (c); and

(e) identifying the test sample as contaminated with mycoplasma if the detection and/or measurement information is different between the test (B) and control (A) samples.

5. A process according to any one of claims 1 to 4 further comprising the step of releasing mycoplasma cellular contents into the culture by treatment of the test sample with a mycoplasma lysis agent.

6. A method as claimed in Claim 5 wherein the lysis agent is a detergent.

7. A method as claimed in Claim 6 wherein the detergent lysis treatment is not capable of lysing bacterial cells.

8. A method as claimed in any of claims 5 to 7 wherein the corresponding control sample is the same as the test sample prior to mycoplasma lysis treatment.

9. A method as claimed in any of claims 1 to 4 wherein the corresponding control sample is the same as the test sample but the obtention of detection/measurement for the test sample activity information is carried out after a time interval following the obtention of detection/measurement information for the control sample.

10. A method as claimed in Claim 9 wherein the time interval is at least approximately 30 minutes.

11. A method according to any of claims 1 to 10 wherein the detecting and/or measuring step comprises detecting and/or measuring ATP.

12. A method according to Claim 11 wherein the ATP is detected and/or measured by a light-emitting reaction.

13. A method as claimed in Claim 12 where the light emitting reaction is a bioluminescent reaction.

14. A method according to any of the preceding claims wherein ADP is added to the test sample prior to the detecting and/or measuring step (ii) or (b).

15. A method according to any of the preceding claims wherein a mycoplasma substrate (MS) reagent is added to the test sample prior to the detecting and/or measuring step (ii) or (b), the MS reagent comprising: acetyl phosphate or a precursor thereof and/or carbamoyl phosphate or a precursor thereof.

16. A method according to Claim 15 wherein the precursor of acetyl phosphate is acetyl-CoA.

17. A method according to Claim 15 wherein the precursor of carbamoyl phosphate is citrulline and/or ammonia.

18. A method according to any of Claims 14 to 17 wherein the control sample is all or an aliquot of the test sample to which a mycoplasma reagent has not been added.

19. A method according to any of Claims 1 to 7, or 9 to 17 wherein the control sample has been shown to be free from mycoplasma by a separate method.

20. A method according to Claim 19 wherein the control sample has been shown to be free from mycoplasma by one or more of PCR testing, DNA fluorescence staining, or mycoplasma culture method.

21. A method as claimed in any preceding claim wherein the sample is a cell-culture sample.

22. A method according to Claim 21 wherein the cells in the cell-culture sample are mammalian cells, preferably adherent cells, such as Vero, MRC5, HUVEC, BSMC, NHEK, MCF-7, AoSMC, A549, HepG2,

FM3A, PC12, ARPE-19, CHO and COS cells, and/or adherent primary cells isolated from animal source.

23. A method according to Claim 21 wherein the mammalian cells in the cell-culture sample grow in suspension, such as K562, U937, HL-60, Cem-7, and Jurkats plus primary cell types such as leukaemic blast cells.

24. A method as claimed in any of claim 21 where the cell culture is a culture of plant cells.

25. A method according to any of Claims 21 to 24 where the cell culture sample is a sample which is derived from a cell culture but is itself substantially free of cellular material.

26. A method according to any of Claims 1 to 20 wherein the sample consists of a cell-free reagent.

27. A method as claimed in Claim 26 where the cell-free reagent is trypsin.

28. A process for treating a cell culture to remove mycoplasma contamination comprising:- treating a mycoplasma contaminated cell culture with an agent to remove and/or destroy mycoplasma; and subsequently testing a sample from the culture for mycoplasma contamination using a method as claimed in any preceding claim; if necessary, repeating the process one or more times until mycoplasma contamination is not detected in the sample.

29. A method of detecting the presence of mycoplasma in a test sample, comprising the following steps:-

(i) providing a test sample;

(ii) without adding an exogenous pyruvate kinase reagent to convert ADP to ATP, detecting and/or measuring ATP in the test sample using a bioluminescent reaction to obtain an ATP and/or light output measurement (B);

(iii) obtaining an ATP and/or light output measurement (A) from a corresponding control sample;

(iv) calculating the ratio $\frac{B}{A}$; and

(v) identifying the test sample as contaminated with mycoplasma in the event that the ratio $\frac{B}{A}$ is greater than one.

30. A method as claimed in any preceding claims wherein the method includes a step of passing the test sample through a filter which retains bacterial cells.

31. Use of acetyl phosphate or a precursor thereof; and/or carbamoyl phosphate or a precursor thereof in a method of detecting contamination of a sample by mycoplasma.

32. A kit for use in the detection of mycoplasma contamination which comprises the following mycoplasma substrate(s):

(i) acetyl phosphate or a precursor thereof and/or

(ii) carbamoyl phosphate or a precursor thereof.

33. A kit as claimed in Claim 32 further comprising ADP at an amount in excess to drive the enzymatic reactions in the direction of ATP formation.

34. A kit as claimed in Claim 32 or 33, further comprising one or more agents for lysing mycoplasma.

35. A kit as claimed in Claim 34, wherein the agents for lysing mycoplasma comprises a detergent.

36. A kit as claimed in any one of Claims 32 to 35 further comprising means for detecting and/or measuring ATP by a light-emitting reaction.

37. A kit as claimed in Claim 36 wherein said means comprise a mycoplasma detection regent (MDR) which includes magnesium acetate, inorganic pyrophosphate, bovine serum albumin, luciferin luciferase, ADP and AMP.

38. A kit as claimed in any one of Claims 37 wherein the reagents are provided in a lyophilised condition.

39. A kit as claimed in any one of Claims 32 to 38 which further comprises a mycoplasma assay buffer (MAB) in which lyophilised reagents can be reconstituted.

40. A kit as claimed in Claim 39 wherein the buffer maintains a pH of approximately 7.5.

41. A kit as claimed in Claim 36 or 37 further comprising a luminometer, which is preferably a hand-held luminometer.

42. A kit as claimed in any one of claims 32 to 41 further comprising a bacterial filter.

43. A kit for detecting mycoplasma contamination of a test sample substantially as described herein, preferably with reference to one or more of the accompanying examples and/or figures.

44. A method of detecting mycoplasma contamination of a test sample substantially as described herein, preferably with reference to one or more of the accompanying examples and/or figures.

45. A method of treating a cell culture to remove mycoplasma contamination substantially as described herein, preferably with reference to one or more of the accompanying examples and/or figures.

# ABSTRACT

## Assay methods and materials

The invention relates to a method of detecting the presence of mycoplasma in a test sample comprising:

(i) providing a test sample; and

(ii) detecting and/or measuring the activity of acetate kinase and/or carbamate kinase in the test sample, the activity being indicative of contamination by mycoplasma.

Figure 1

FIGURE 1

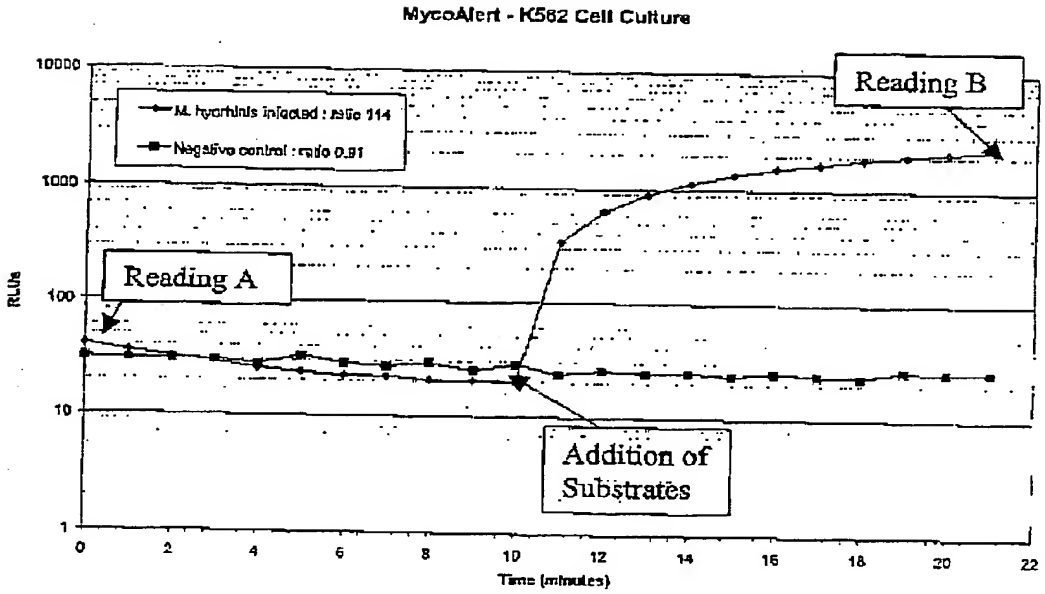

MycoAlert - K562 Cell Culture
M. hyorhinis infected : ratio 114
Negative control : ratio 0.91
Reading A
Reading B
Addition of Substrates
RLUs
Time (minutes)
10000
1000
100
10
1
0
2
4
6
8
10
12
14
16
18
20
22

**FIGURE 2**

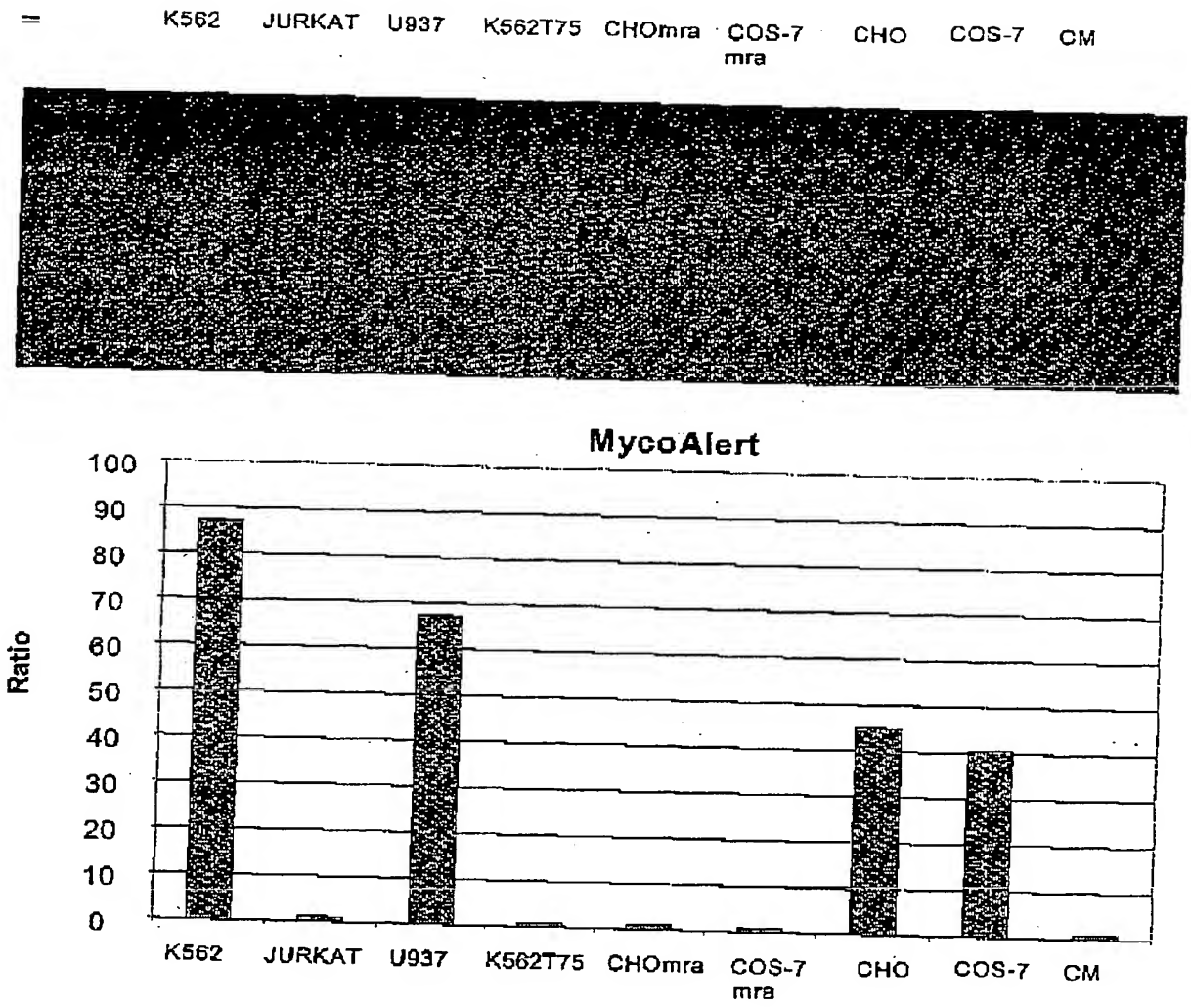

=
K562
JURKAT
U937
K562T75
CHOmra
COS-7 mra
CHO
COS-7
CM
MycoAlert
Ratio
100
90
80
70
60
50
40
30
20
10
0
K562
JURKAT
U937
K562T75
CHOmra
COS-7 mra
CHO
COS-7
CM

**FIGURE 3**

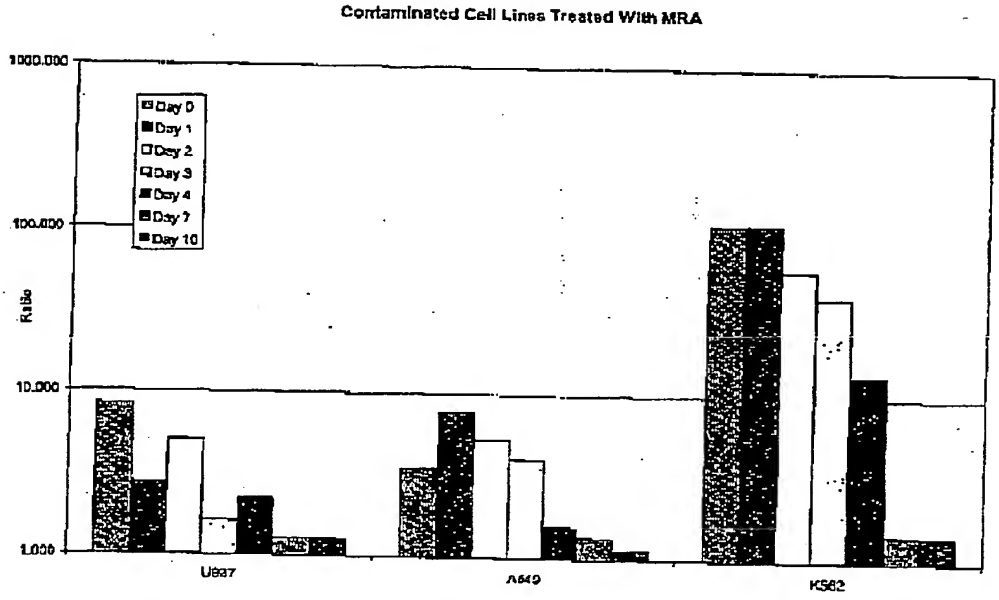

Contaminated Cell Lines Treated With MRA
1000.000
100.000
10.000
1.000
Ratio
Day 0
Day 1
Day 2
Day 3
Day 4
Day 7
Day 10
U937
A549
K562

FIGURE 4

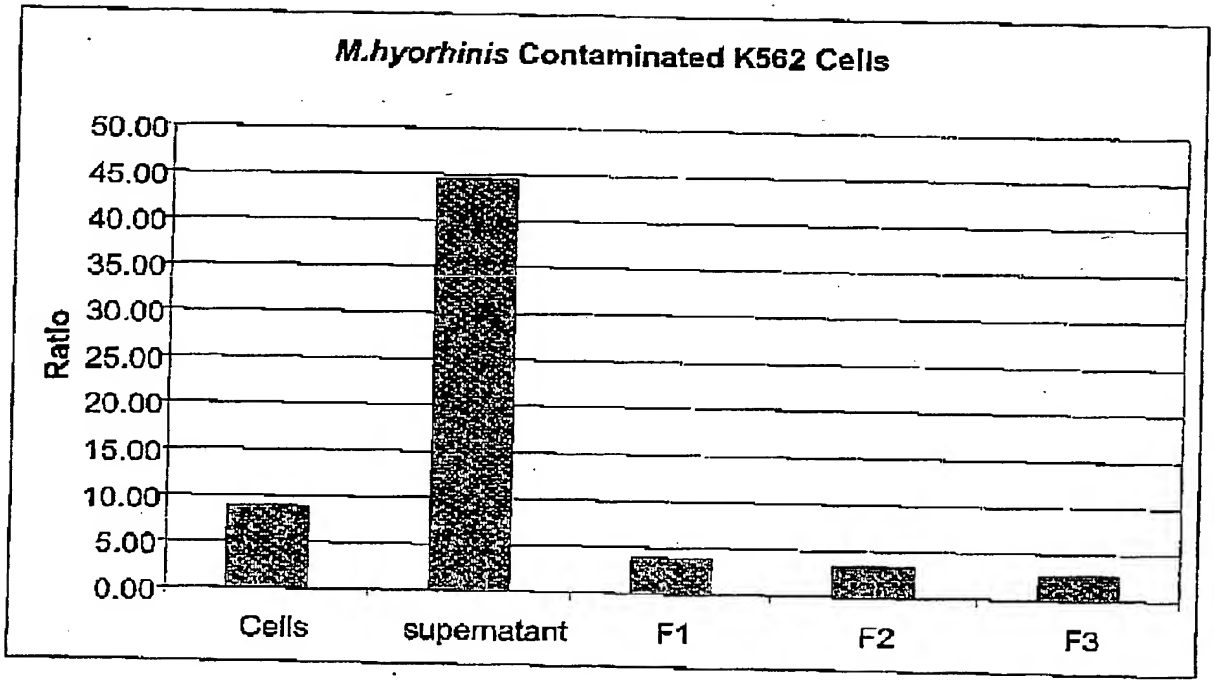

M.hyorhinis Contaminated K562 Cells
Ratio
50.00
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Cells
supernatant
F1
F2
F3

**FIGURE 5**

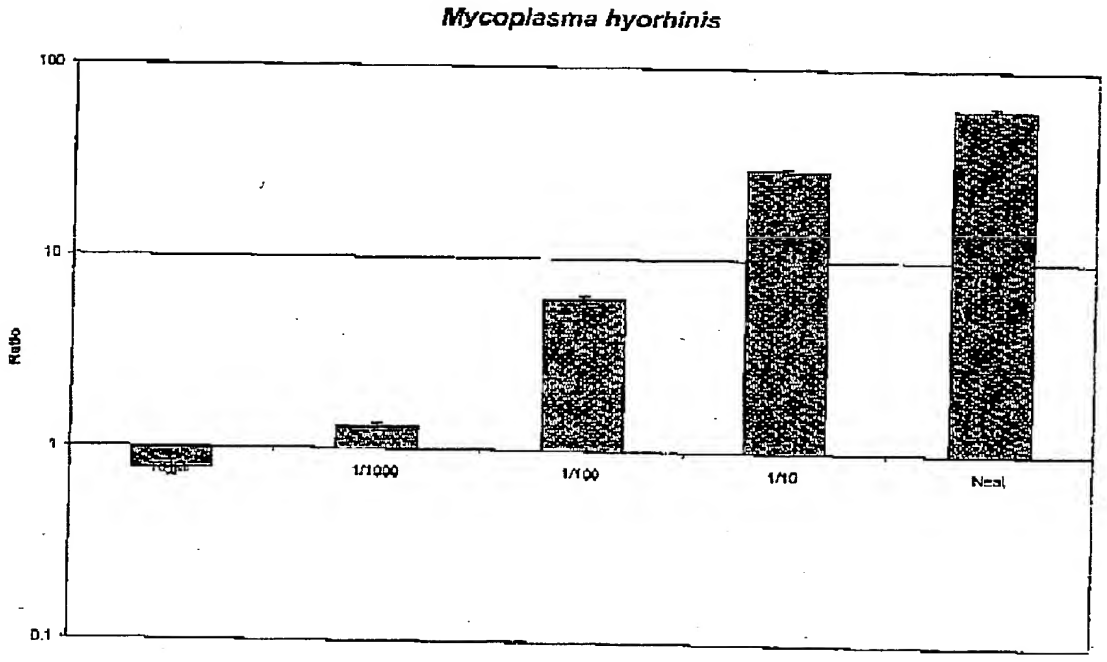


**FIGURE 6**

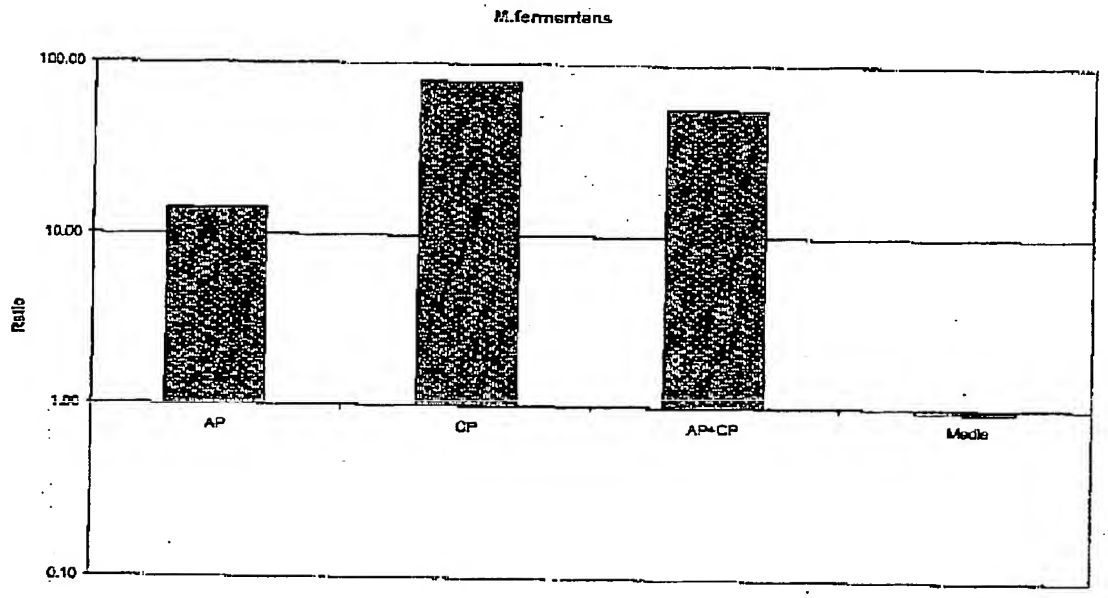


FIGURE 7

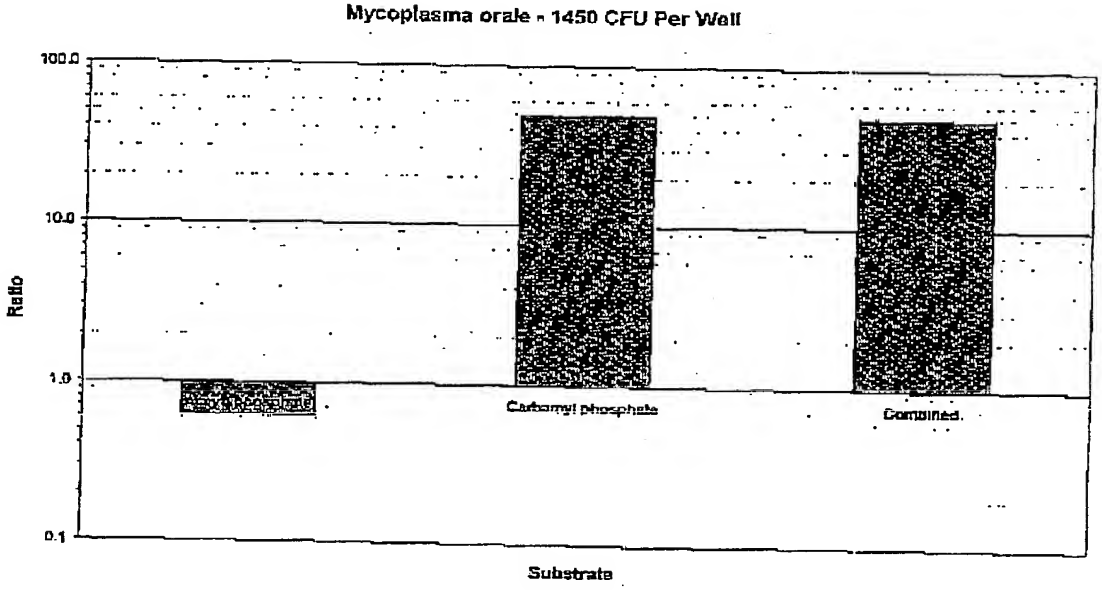



FIGURE 8

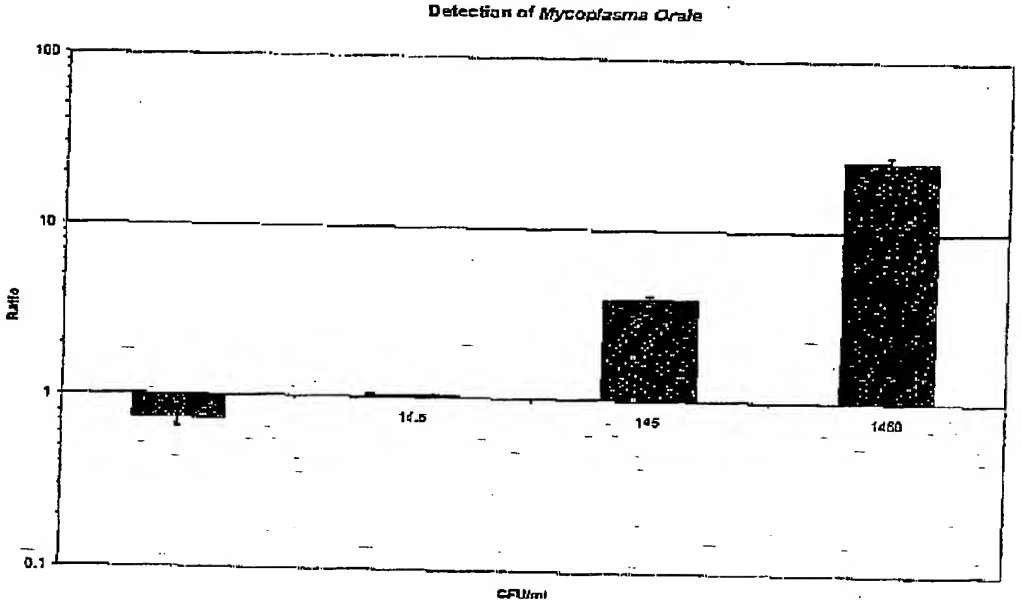


FIGURE 9

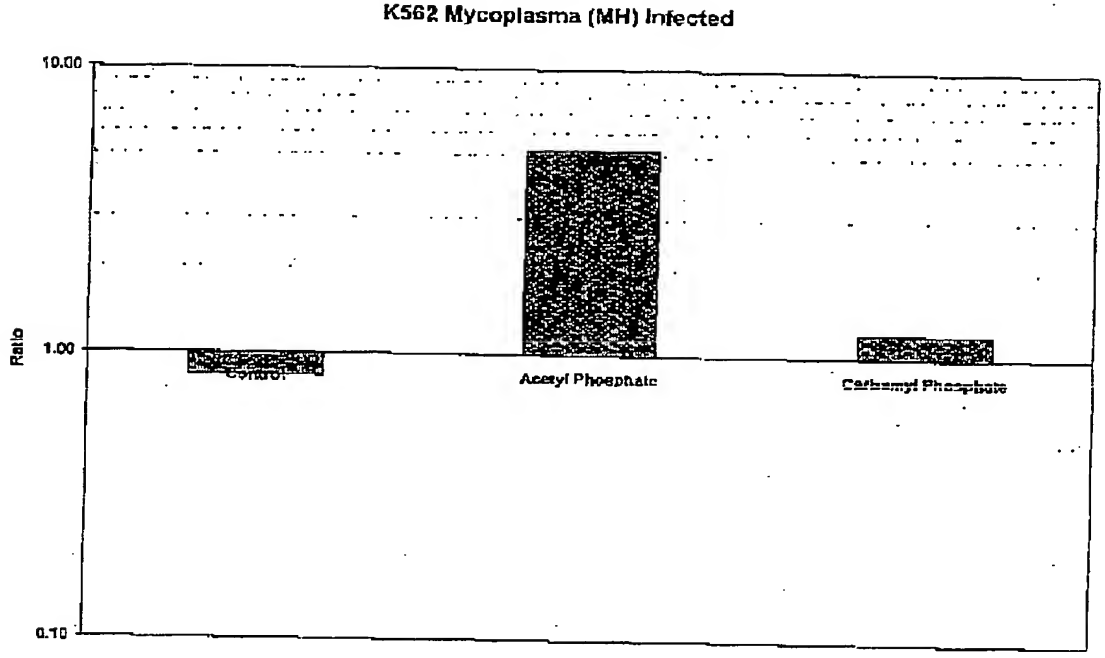

K562 Mycoplasma (MH) Infected
10.00
1.00
0.10
Ratio
Acetyl Phosphate
Carbamyl Phosphate

FIGURE 10

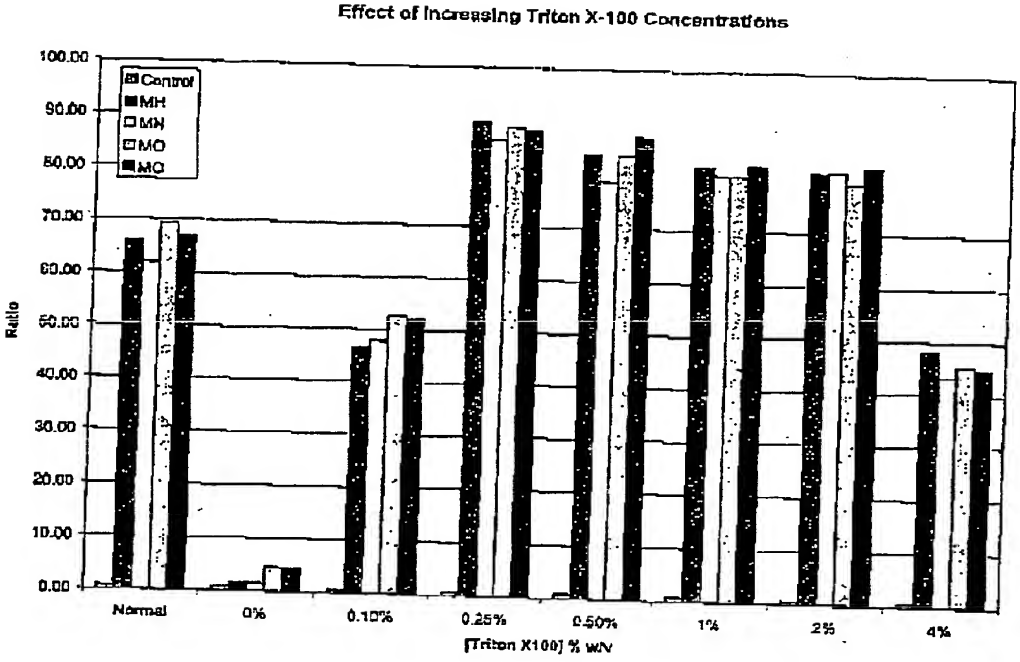
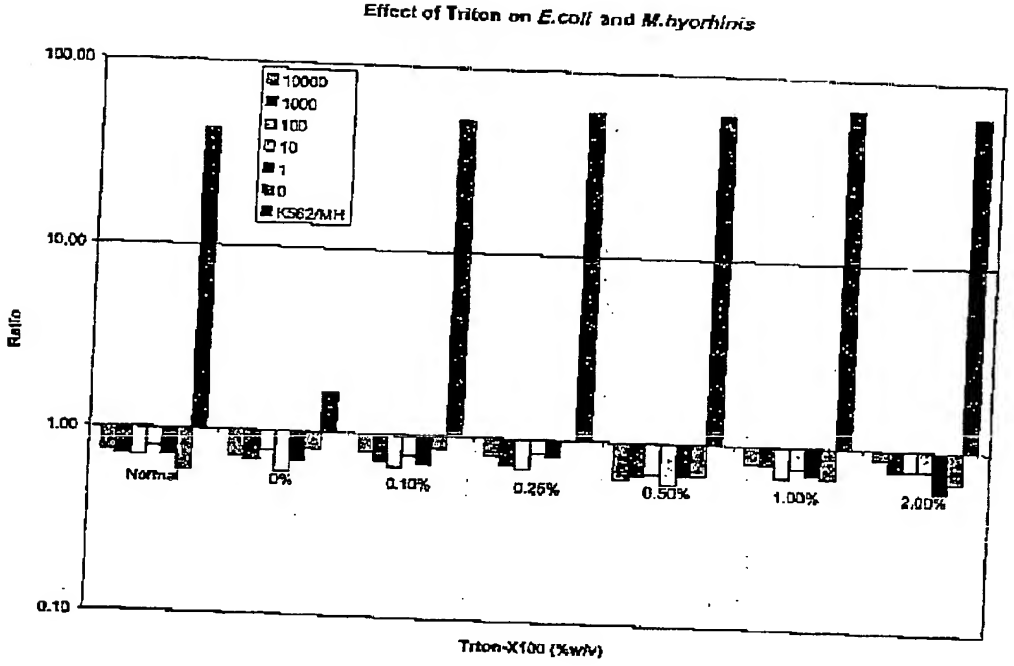

Effect of Increasing Triton X-100 Concentrations
Control
MH
MN
MO
MO
Ratio
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
Normal
0%
0.10%
0.25%
0.50%
1%
2%
4%
[Triton X100] % w/v

FIGURE 11


Effect of Triton on E.coli and M.hyorhinis
100.00
10.00
1.00
0.10
Ratio
10000
1000
100
10
1
0
K562/MH
Normal
0%
0.10%
0.25%
0.50%
1.00%
2.00%
Triton-X100 (%w/v)